Species	:	Selenastrum capricornutum (Algae)
Endpoint	:	Cell density, biomass and growth rate
Exposure period	:	96 hour(s)
Unit	:	mg/l
NOEC	:	0.170 mg EMCA/L nominal
EC50	:	= .317 calculated based on nominal concentrations
Limit test	:	
Analytical monitoring	:	yes
Method	:	OECD Guide-line 201 "Algae, Growth Inhibition Test" USEPA, Toxic Substance Control Act Test Guidelines; Final Rule. 40 CFR Part 797.1050. Subpart B Federal register.
Year	:	2002
GLP	:	yes
Test substance	:	other TS: >98.5% pure

Method :

Test Vessels
Test vessels were sterilized 250-mL borosilicate Erlenmeyer flask with Shimadzu closure each containing 100 mL test medium. Each flask was labeled with a unique number for identification purposes.

Culture and Test Medium
The growth and test medium used was that designed for the EPA Algal Assay Bottle Test, referred to as algal assay medium (AAM).

Algal Inoculum
The algal inoculum was prepared from a 4-day old stock culture of Selenastrum capricornutum Printz. A Coulter Multisizer was used to determine the algal density of the stock culture. This evaluation determined the aliquot of the culture required so that each test vessel would contain approximately 10,000 cells/mL. The day 0 average cell density was calculated using data from each individual replicate; the average day-0 cell density was 11,000 cells/mL.

Dose Level Selection
The dose levels selected for evaluating the effects of EMCA on the growth of *Selenastrum capricornutum* were based on the results of a probe study. The probe study was conducted in January 2002 using five nominal concentrations of 0.1, 1, 10, 100, and 1000 mg nominal EMCA/L. Test concentrations and the media control groups were set in duplicate. The probe was carried out aseptically for four days. The 4-day EC50 value was between 1 and 0.1 mg nominal/L and the no-observable-effect-concentration was 0.1 mg nominal/L. The information derived from this test was used to set the range of concentrations for the definitive study.

Test Solutions
The definitive study was conducted under static exposure conditions with test vessels (160-mL borosilicate serum vials, six replicate vessels per dose) containing AAM dosed at target EMCA concentrations of 0.058, 0.082, 0.118, 0.168, 0.240, 0.343, 0.490, 0.7 and 1 mg EMCA/L AAM; the test design also included an algal medium negative control group.

Test Solution Preparation
A 10 mg nominal/L primary stock solution was prepared by directly pipetting a 8.7 µL aliquot of EMCA in a 1-L sterile volumetric flask and diluting to volume with AAM. The resulting solution was observed to be clear and void of undissolved test material (e.g., precipitate). The test solutions were prepared from dilutions of the 10 mg/L primary stock solution as detailed in Table 1.

Table 1: Dose Solution Preparation

Nominal Stock Concentration (mg EMCA/L	Volume of Stock Used (mL)	Diluted to (mL) with AAM	Nominal EMCA Concentration (mg/L)
10	100	1000	1
10	70	1000	0.7

10	49	1000	0.49
10	34	1000	0.343
10	24	1000	0.240
10	17	1000	0.168
10	12	1000	0.118
10	8.2	1000	0.082
10	5.8	1000	0.058

Chemical Analysis
Analytical verification of dose solutions was not performed.

Exposure Phase
The definitive test was conducted between 29 January 2002 and February 2 2002 using nine target exposure concentrations of 0.058, 0.082, 0.118, 0.168, 0.240, 0.343, 0.490, 0.7 and 1 mg EMCA/L, set in a geometric series using a progression factor of ~2.0. The test design also included an algal assay medium control (negative control). Six replicates were set of each test concentration, and each test flask was then inoculated with approximately 11,000 cells/mL. The media control was set with seven replicates, six inoculated with algal culture and one without (counting blank). The exposure phase was carried out aseptically under static conditions for 4 days. The test flasks were placed in an incubator (Environ-Shaker Model 3597, Lab-Line Inc., Melrose, Illinois) thermostated at 24 ± 2 °C with continuous light at approximately 8000 ± 1600 lux.

Physical Analysis
The pH at initiation of the test for each bulk solution and those read and discarded on day 3 of the study were inadvertently not recorded. The pH for each individual vessel at the termination of the study was recorded. The incubator temperature was continuously monitored with a temperature probe placed in a representative vessel within the incubator. The light intensity was monitored daily at positions corresponding to the test flasks in the incubator.

Algal Density Determinations
Algal cell densities of the initial inoculum and test cultures were determined by electron particle counting using a Coulter Multisizer. Total cell counts were determined after 72 hours of exposure for the last three vessels in each exposure group and the control group and the first three vessels for each exposure group and the control group following 96 hours of exposure. Cells were cumulatively counted at a lower threshold equivalent spherical diameter of approximately 2.7 μm to a higher threshold equivalent spherical diameter of approximately 8.6 μm. A media correction blank was compensated for in the cell density calculations.

Statistical Analysis
The results (study endpoints) of the study were evaluated based on nominal EMCA concentrations. The endpoints measured were percent inhibition of growth, growth rate and algal growth (total cell count/mL). The EbC50 value (the concentration that inhibits the growth of this algal species to 50% of the test population, when compared to the control population) was calculated by regression of the differences in area under the growth curves for each dose group compared to the control against the log of the concentrations for days 3 and 4. The ErC50 value (the concentration that inhibits the growth rate of this algal species to 50% of the test population, when compared to the control population) was calculated by regressing the percent reduction in average growth rate for each dose group compared to the control group against the natural logarithm of the concentrations for the 0 to 72 hour exposure period and the 0 to 96 hour exposure period.

The EC25 and EC50 values (those concentrations that limited growth of this algal species to 25%, and 50% of the test population, respectively, when compared to the control population) for algal growth were determined by a least squares linear regression of algal cell counts against the log of the concentration on day 3. The no-observed- effect concentrations (NOECs) for algal growth were calculated using the analysis of variance and Dunnett's test.

Experimental dates: 01/29/2002 to 02/02/2002

Result : Test Conditions

Temperature (°C), light intensity (lux), and pH data ranges observed during the four-day exposure phase are summarized in Table 3. Temperatures during the exposure period averaged (± standard deviation) 24.2 ± 0.4 °C (23.7 °C – 24.8 °C), light intensity averaged 7540 ± 335 lux (6430 lux – 8230 lux), and pH values ranged 7.0 to 7.3 without algae, and from 8.4 to 10.3 with algae during the test period.

Biological Data

The effects of EMCA on algal growth after 72 hours of exposure, relative to the control group, ranged from 2.9% stimulation of growth at 0.082 mg/L to 98.9% inhibition of growth at 1.00 mg/L. Effects of EMCA on algal growth after 96 hours of exposure, relative to the control group, ranged from 11.2% stimulation of growth at 0.168 mg/L to 96.5% inhibition of growth at 1.00 mg/L.

Table 2 lists the mean total algal cell counts/mL for each exposure concentration (three replicates/concentration), and the standard deviations and coefficients of variation for exposure days 3, and 4 of the study. All results are expressed in terms of nominal EMCA concentrations.

Table 2: Summary of Growth Data Expressed as Algal Cells/mL:

Nominal EMCA Concentration in mg/L	Statistical Measure	Cells Density (Algal Cells/mL) – Day 3	Cells Density (Algal Cells/mL) – Day 4
AAM Control	Mean	1025049	1163907
	STD	50637	64596
	CV (%)	4.9	5.5
0.058	Mean	1025049	1271694
	STD	50637	34135
	CV (%)	4.9%	2.7%
0.082	Mean	1084466	1333112
	STD	51449	64052
	CV (%)	4.7%	4.8%
0.118	Mean	1044880	1286472
	STD	39118	15127
	CV (%)	3.7%	1.2%
0.240	Mean	118363*	886330*
	STD	25866	70842
	CV (%)	21.9%	8.0%
0.343	Mean	118363*	531521*
	STD	25866	187520
	CV (%)	21.9%	35.3%
0.490	Mean	57713*	64585*
	STD	1640	12309
	CV (%)	2.8%	19.1%
0.700	Mean	11697*	69679*
	STD	1219	20013
	CV (%)	10.4%	28.7%
1.00	Mean	11631*	41577*
	STD	212	6333
	CV (%)	1.8%	15.2%

STD: Standard deviation.
CV: Coefficient of variation.
*: Significant difference from the combined controls; p<=0.05 one tailed Dunnett's t-test on raw data.

The calculated 72-hour ErC50 value for growth rate, based on nominal EMCA concentrations, was 0.366 mg/L. The calculated 96-hour ErC50 value for growth rate, based on nominal EMCA concentrations, was 0.632 mg/L.

The calculated 72-hour EbC50 value for inhibition of growth, based on nominal EMCA concentrations, was 0.247 mg/L (95% confidence interval – 0.103 mg/L – 0.593 mg/L). The regression equation for day 3 was percent inhibition = 112.2 + 102.5 *(Log Concentration), with an R2 value of 0.898.

The calculated 96-hour EbC50 value for inhibition of growth, based on nominal EMCA concentrations, was 0.261 mg/L (95% confidence interval – 0.107 mg/L – 0.639 mg/L). The regression equation for day 4 was percent inhibition = 109.8 + 102.5*(Log Concentration), with an R2 value of 0.894.

The calculated 72-hour EC10 value for algal growth, based on nominal EMCA concentrations, was 0.101 mg/L (95% confidence interval – 0.049 mg/L – 0.207 mg/L).

The calculated 72-hour EC50 value for algal growth, based on nominal EMCA concentrations, was 0.251 mg/ L (95% confidence interval – 0.124 mg/L – 0.508 mg/L).

The calculated 72-hour EC90 value for algal growth, based on nominal EMCA concentrations, was 0.622 mg/ L (95% confidence interval – 0.303 mg/L – 1.29 mg/L). The regression equation for day 3 based on nominal EMCA concentrations was total cell counts = -114865 – 1068393*(Log Concentration), with an R2 value of 0.894. The statistically derived 72-hour NOEC, based on nominal EMCA concentrations, was 0.120 mg/L.

The calculated 96-hour EC10 value for algal growth, based on nominal EMCA concentrations, was 0.128 mg/L (95% confidence interval – 0.051 mg/L – 0.319 mg/L). The calculated 96-hour EC50 value for algal growth, based on nominal EMCA concentrations, was 0.317 mg/ L (95% confidence interval – 0.127 mg/L – 0.787 mg/L). The calculated 96-hour EC90 value for algal growth, based on nominal EMCA concentrations, was 0.787 mg/ L (95% confidence interval – 0.309 mg/L – 2.005 mg/L). The regression equation for day 4 based on nominal EMCA concentrations was total cell counts = -6661 – 1213284*(Log Concentration), with an R2 value of 0.835. The statistically derived 96-hour NOEC, based on nominal EMCA concentrations, was 0.170 mg/L.

Reliability : (1) valid without restriction
1 (guideline, GLP study)